Molecular confirmation of Dicrocoelium dendriticum in the Himalayan ranges of Pakistan

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Abstract

Lancet liver flukes of the genus *Dicrocoelium* (Trematoda: Digenea) are recognised parasites of domestic and wild herbivores. The aim of the present study was to confirm the species identity of Dicrocoeliid flukes collected from the Chitral valley in the Himalayan ranges of Pakistan. The morphology of 48 flukes belonging to eight host populations was examined; but overlapping traits prevented accurate species designation. Phylogenetic comparison of published *D. dendriticum* ribosomal cistron DNA, and cytochrome oxidase-1 (COX-1) mitochondrial DNA sequences with those from *D. chinensis* was performed to assess within and between species variation and re-affirm the use of species-specific single nucleotide polymorphism markers. PCR and sequencing of 34 corresponding fragments of ribosomal DNA and 14 corresponding fragments of mitochondrial DNA from the Chitral valley flukes, revealed 10 and 4 unique haplotypes, respectively. These confirmed for the first time the molecular species identity of Pakistani lancet liver flukes as *D. dendriticum*. This work provides a preliminary illustration of a phylogenetic approach that could be developed to study the ecology, biological diversity, and epidemiology of Dicrocoeliid lancet flukes when they are identified in new settings.

Keywords: Dicrocoeliid lancet flukes; *Dicrocoelium dendriticum*; morphological traits; ribosomal and mitochondrial markers.

1. Introduction

Dicrocoeliid liver flukes can infect the bile ducts of a variety of wild and domesticated mammals and humans around the globe. Three species of the genus *Dicrocoelium*, namely *Dicrocoelium dendriticum*, *Dicrocoelium hospes* and *Dicrocoelium chinensis* have been described as causes of dicrocoeliosis in domestic and wild ruminants [1]. Among these, *D. dendriticum* is the most common and is distributed throughout Europe, Asia, North and South America, Australia, and North Africa [2]. The main economic impact of dicrocoeliosis in livestock is due to the rejection of livers with cholangitis from slaughtered animals at meat inspection [3]. However, in severe infections, affected animals may show clinical signs including poor food intake, ill thrift, poor milk production, alteration in faecal consistency, photosensitisation and anaemia [4, 5].

The life history of *Dicrocoelium* spp. is indirect and may take at least six months to complete. Monoecious and both sexually reproducing and self-fertilising adults are found in the bile ducts. Eggs containing fully-developed miracidia are shed in faeces and must be eaten by land snails before hatching. Miracidia undergo asexual replication and development into cercariae, which then escape from the snails in their slime trails, and are eaten by ants. One cercaria migrates to the head of the ant and associates with the sub-oesophageal ganglion; while up to about 50 cercariae encyst in the gaster as metacercariae [6]. The larval stage that develops in the ant's head alters its behaviour, making it cling to herbage and increasing the probability of its being eaten by a herbivorous definitive host. Larval flukes migrate to the liver via the biliary tree and develop to adults in the bile ducts [4]. Several species of land snails and ants are known to be intermediate hosts within the same geographical location [7]; nevertheless the geographical distribution of *Dicrocoelium* spp. is constrained by the precise conditions required for completion of the parasite's life history.

Molecular methods amplifying fragments of nuclear ribosomal genes and their internal transcribed spacers, or mitochondrial loci DNA [8, 9, 10, 11, 12, 13] have been developed for Dicrocoeliid parasites; but as with all molecular diagnostic tools, these depend on accurate morphological speciation of the reference materials. These methods are adaptable to demonstrate genetic variability and phylogeny, and have been applied to epidemiological studies of various trematode parasite species affecting ruminant livestock [14, 15, 16, 17]. However, in the study of Dicrocoeliid genera, the value of phylogenetic studies to detect intraspecific variation is limited by the availability of comparable sequence data.

The Chitral valley has economic and strategic importance as a route of human and animal movements to and from south-east Asia through what is known as the China-Pakistan economic corridor. The average elevation is 1,500 m and the daily mean temperature ranges from 4.1°C to 15.6°C, creating an arid environment with only patchy coniferous tree cover, and providing habitats that are mostly hostile to many snail species. Moving north from Peshawar over the Lowari Pass into Chitral valley the snail fauna changes completely [18], potentially creating isolated habitats for *Dicrocoelium* spp. flukes in this region as compared to other parts of Pakistan. The economics of livestock production is marginal in this region,

hence any improved understanding of any potential production limiting disease, such as dicrocoeliosis is important.

In this study, we describe variability in morphological features of Dicrocoeliid parasites recovered from slaughtered sheep in the Chitral valley, along with corresponding genomic sequence data for fragments of ribosomal DNA and cytochrome oxidase-1 (COX-1) mtDNA. Our aim was to use these data to describe any possible phylogenetic relationships within and between Pakistani *D. dendriticum* and the limited number of other *Dicrocoelium* spp. for which matching sequence data are publicly available.

2. Materials and Methods

2.1. Fluke collection

A convenience sampling method was used to examine the livers of a total of 144 sheep slaughtered at four abattoirs in the Chitral valley of the Kyber Pakhtunkhwa province of Pakistan. These comprised of 68 from Booni, 26 from Torkhow, 33 from Mastuj and 17 from the Laspoor valley. Overall, 639 typical adult Dicrocoelid flukes (25 to 50 flukes per liver) were recovered from the livers of 16 infected sheep. The flukes were washed with phosphate-buffered saline (PBS) to remove adherent debris and fixed in 70% ethanol for subsequent morphometric and molecular characterisation.

2.2. Morphological examination

Six adult flukes were selected from each of the livers of four of the 12 infected sheep from Booni (Pop-1, Pop-2, Pop-3, Pop-4), the one infected sheep from Laspoor (Pop-5), the two infected sheep from Mastuj (Pop-6, Pop-7) and the one infected sheep from Thorkhow (Pop-8) and stained for morphometric analysis. The flukes were fixed between two glass slides in formalin-acetic acid alcohol solution, stained with hematoxylin (Sigma-Aldrich) and mounted in Canada balsam. Standardised measurements were taken and orientation of the testes was noted as previously described [19, 9].

2.3. PCR amplification and sequence analysis of ribosomal and mitochondrial DNA

Genomic DNA was successfully extracted from 34 individual adult flukes (3 to 6 flukes per animal) from the livers of the same eight sheep (Pop-1 to Pop-8), using a standard phenol-chloroform method [20]. 1,152 bp of the rDNA cistron, comprising of fragments of the ITS1, 5.8S, ITS2 and 28S flanking region, were amplified by using two sets of universal primers (Table 1) as previously reported [13]. A 215 bp fragment of cytochrome c oxidase subunit-1 (COX-1) mtDNA was amplified using newly developed forward and reverse primers, designed with reference sequences downloaded from NCBI using the 'Primers 3' online tool (Table 1). The 25 µl PCR reaction mixtures consisted of 2 µl of PCR buffer (1X) (Thermo Fisher Scientific, USA), 2 µl $MgCl_2$ (25 mM), 2 µl of 2.5 mM dNTPs, 0.7 µl of primer mix (10 pmol/µl final concentration of each primer), 2 µl of gDNA, and 0.3 µl of Taq DNA polymerase (5 U/µl) (Thermo Fisher Scientific, USA) and 16 µl ddH_20. The thermocycling conditions were 96^oC for 10 min followed by 35 cycles of 96^oC for 1 min, 60.9^oC (BD1-F-rDNA/ BD1-R-rDNA), or 61.4^oC

(Dd-F-rDNA/ Dd-R-rDNA), or 55°C (D-F-cox11/ D-R-cox11) for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min.

PCR products were cleaned using a WizPrepTM Gel/PCR Purification Mini kit (Seongnam 13209; South Korea) and submitted for sequencing of both strands on an Applied Biosystems 3730Xl genetic analyser (Eurofins Genomics LLC), using the same amplification primers. Both strands of rDNA, and COX-1 sequences from each fluke were assembled, aligned and edited to remove primers from both ends using Geneious Pro 5.4 software [21]. The sequences were then aligned with previously published NCBI GenBank rDNA and COX-1 sequences of *D. dendriticum* and *D. chinensis*. All sequences in the alignment were trimmed based on the length of the shortest sequence available that still contained all the informative sites. Sequences showing 100% base pair similarity were grouped into single haplotypes using the CD-HIT Suite software [22].

2.4. Molecular phylogeny of the rDNA and COX-1 data sets

The generated haplotypes were imported into MEGA 7 [23] and used to determine the appropriate model of nucleotide substitution. Molecular phylogeny was reconstructed from the rDNA and COX-1 sequence data by the Maximum Likelihood (ML) method. The evolutionary history was inferred by using the ML method based on the Kimura 2-parameter model for rDNA and Hasegawa-Kishino-Yano model for the COX-1 locus. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood values. All positions containing gaps and missing data were eliminated. There were totals of 698 bp, and 187 bp in the final datasets of rDNA and COX-1, respectively. A split tree was created in the SplitTrees4 software by using the UPGMA method in the HKY85 model of substitution. The appropriate model of nucleotide substitutions for UPGMA analysis was selected by using the jModeltest 12.2.0 program.

3. Results

3.1. Fluke morphometric characteristics

Forty-eight hematoxylin-stained liver flukes were examined. The flukes were all lanceolate, with flattened bodies, and were 5.65 to 8.7 mm in length and 1.3 to 2.2mm in width. The overlapping ranges of morphometric measurements and orientation of the testes of the examined flukes did not allow for accurate species differentiation between *D. dendriticum* and *D. chinensis* (Fig. 1).

3.2. Molecular confirmation of Dicrocoelium *species identity*

3.2.1. Ribosomal DNA haplotypes

The rDNA sequences of each of 34 flukes from which genomic DNA was successfully extracted were aligned with 12 sequences of *D. dendriticum* and 18 sequences of *D. chinensis* (Supplementary Data S1) available on the Mendeley database and trimmed to 698 bp length. This included 4 informative sites to describe intraspecific variation within *D. dendriticum* and 6 sites to describe intraspecific variation within *D. chinensis*. The alignment confirmed 21 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 2); this allowed the molecular species identity of each of the 34 flukes to be confirmed as *D. dendriticum*.

The 12 *D. dendriticum* and 18 *D. chinensis* sequences from the public database were examined along with the 34 *D. dendriticum* rDNA sequences from the present study. Sequences showing 100% base pair similarity were grouped into single haplotypes generating 19 unique *D. dendriticum* and 4 unique *D. chinensis* haplotypes (Supplementary Data S1). A ML tree was constructed from the 23 rDNA haplotypes to examine the evolutionary relationship between *D. dendriticum* and *D. chinensis*. The phylogenetic tree shows that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Fig. 2a). Ten haplotypes generated from the 34 rDNA sequences from the Chitral valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China showed some common origins and close relationships (Table 3), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*. The rDNA haplotypes generated from the eight Pakistani *D. dendriticum* populations are shown in a split tree (Fig. 3); albeit there are too few data to draw any conclusions.

3.3.2. Mitochondrial COX-1 haplotypes

Unfortunately, COX-1 sequences of sufficient quality were generated from only 14 of the 34 flukes from which genomic DNA was successfully extracted. These were aligned with 56 *D. dendriticum* sequences and 11 *D. chinensis* sequences available on the public NCBI Mendeley database (Supplementary Data S2) and trimmed to 187 bp length. This included 7 informative sites of intraspecific variation within *D. dendriticum* and 3 sites of intraspecific variation within *D. chinensis*. The alignment confirmed 12 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 2), and allowed the molecular species identity of the 14 flukes to be re-affirmed as *D. dendriticum*.

The 56 COX-1 *D. dendriticum* and 11 *D. chinensis* sequences from the public database were examined along with the 14 *D. dendriticum* mtDNA sequences from the present study. Sequences showing 100% base pair similarity were grouped into single haplotypes generating 8 unique *D. dendriticum* and 3 unique *D. chinensis* haplotypes (Supplementary Data S2). A ML tree was constructed from the 11 unique COX-1 haplotypes to examine the evolutionary relationship between *D. dendriticum* and the other liver flukes. The phylogenetic tree re-affirms that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Fig. 2b). Four haplotypes generated from the 14 COX-1 sequences from the Chitral valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China, Shiraz province of Iran and Japan showed some common origins and close relationships (Table 3), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*.

4. Discussion

Dicrocoeliid liver flukes have previously been reported in Himalayan India, [24], China [25, 26] and Iran [27, 28]; but there are only anecdotal and unconfirmed reports from Pakistan [29]. The molecular methods used in the present study confirm for the first time the species identity of *D. dendriticum* lancet flukes collected from abattoirs in the Chitral valley. Knowledge of the parasite species infecting livestock in any geographical region is of particular importance when considering effective and sustainable control strategies. Our confirmation of *D. dendriticum* in Pakistan highlights the need for better understanding of aspects of the parasite's biology, such as the identification of the species of snail and ant that may act as competent intermediate hosts.

Previous studies have shown the value of morphological and molecular-based methods for the accurate species differentiation between *D. dendriticum* and *D. chinensis* [9, 11, 12, 13]. The specimens collected from the Chitral valley were identified as Dicrocoeliid genus, but overlapping morphological traits prevented their accurate species designation.

Our analyses of ribosomal cistron and mitochondrial COX-1 loci sequences of *D. dendriticum* and *D. chinensis* from the public database showed consistent inter-specific variations. Twenty-one discriminatory ribosomal cistron SNPs and 12 discriminatory COX-1 SNPs were identified, allowing practical differentiation of the Dicrocoeliid family as previously described [11]. *D. hospes* was not included in our analyses, because directly comparable sequence data are not publicly available. The ML tree that was generated shows separate clades of *D. dendriticum* and *D. chinensis*, hence in the case of co-infections, molecular differentiation between each of the species is possible. The Chitral valley lancet flukes were all *D. dendriticum*, irrespective of their morphological identity. Morphological traits may be influenced by: the stages of maturation of the flukes at the time of collection; the intensity of infection; factors linked to the host species; normal biological variation; or errors introduced during processing. Furthermore, the published morphological keys used for *D. chinensis* are based on parasites recovered from sika deer, whereas those for *D. dendriticum* are based on parasites recovered from sheep, albeit with no significant intraspecific variation reported from cattle derived parasites [9]. Consideration of these factors highlights the complementary value of morphological and molecular and methods in fluke species identification.

Our analyses of ribosomal cistron and mitochondrial COX-1 loci sequences of *D. dendriticum* and *D. chinensis* from the public database showed consistent intra-specific variations. Ten haplotypes in the ribosomal cistron fragment and four in the mitochondrial COX-1 sequences from the Chitral valley of Pakistan were then used to analyse gene flow. Unfortunately, comparable sequence data for European and north American *D. dendriticum* populations were unavailable in the public database for analysis. There were both unique and common haplotypes in each *D. dendriticum* population from the Chitral valley, some of which were also present in populations form China, Iran and Japan. There are insufficient data on which to base firm conclusions; hence further studies based on larger population sizes, and using for example next generation methods as described for *Calicophoron daubneyi* rumen flukes and *F. gigantica* liver flukes [16, 17] are needed to describe gene flow and the role of animal movement in the spread of *D. dendriticum*.

Our findings support the potential for the development of population genetics tools to improve understanding of the molecular evolutionary biology and phylogenetics of *D.*

dendriticum. This is needed to study changing epidemiology of the parasite, potentially arising as a consequence of changing management and climatic conditions, as previously described using a panel of microsatellites and a COX-1 mtDNA sequence marker [30].

Figures and Tables

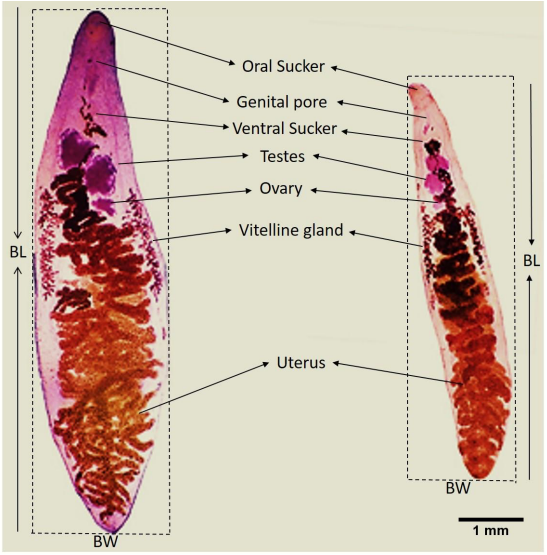

Fig. 1: Light micrograph of hematoxylin-stained flukes from the present study. The bodies are pointed at both ends and semi-transparent, with a pair of lobate testes behind the ventral sucker. The ovary is small, and the uterus has both ascending and descending limbs and white vitellaria. The morphometric measurements and orientation of the testes of 23 flukes were similar to those shown on the left, and the equivalent features of 25 flukes were similar to those shown on the left.

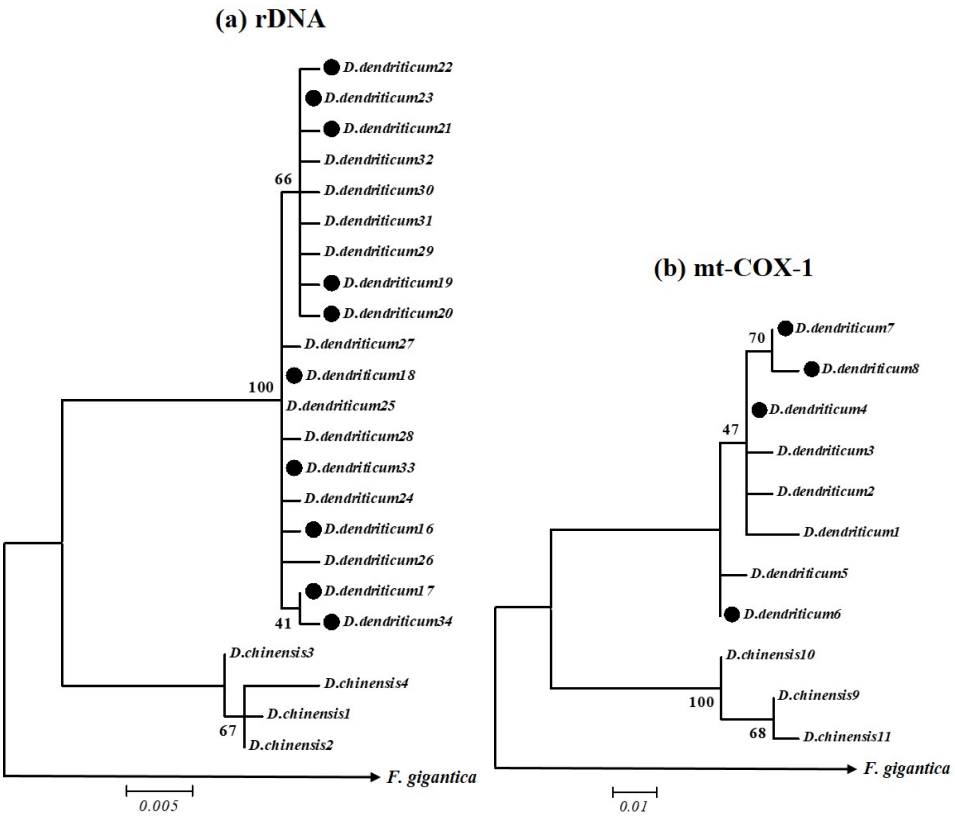

Fig. 2: Maximum-likelihood trees were obtained from the rDNA and COX-1 mtDNA sequences of *D. dendriticum* and *D. chinensis*. (a) Thirty-eight unique rDNA haplotypes. (b) Thirty-six unique COX-1 mtDNA haplotypes. The haplotype of each species is identified with the name of the species and black circles indicate *D. dendriticum* haplotypes originating from the Chitral valley of Pakistan.

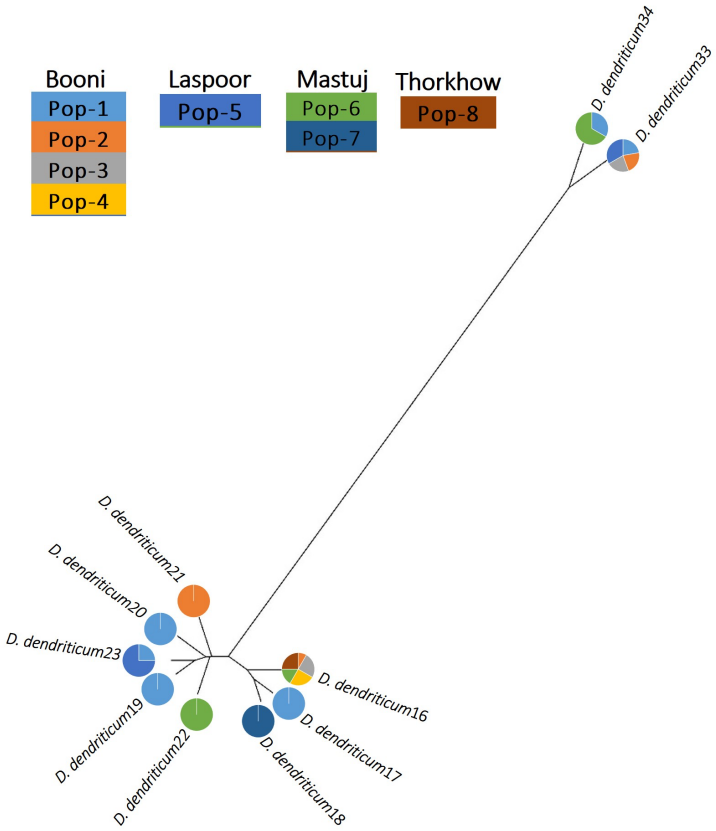

Fig. 3: Split tree of 10 rDNA haplotypes generated from eight Pakistani *D. dendriticum* populations. The tree was constructed with the UPGMA method in the HKY85 model of substitution in the SplitsTrees4 software. The pie chart circle represent the haplotype distribution amongst each of the eight populations. The colour of each haplotype circle represents the percentage of sequence reads generated per population.

Primer name	Sequences (5'-3')	
BD1-F-rDNA	GTCGTAACAAGGTTTCCGTA	
BD2-R-rDNA	TATGCTTAAATTCAGCGGGT	(Gorjipoor *et al*., 2015)
Dd-F-rDNA	ATATTGCGGCCATGGGTTAG	
Dd-R-rDNA	ACAAACAACCCGACTCCAAG	(Gorjipoor *et al*., 2015)
D-F-cox11	TGAGGTCTTGGATCGTGTTA	
D-R-cox11	AAACCACCAACTCACCAAAC	

Table 1. Primer sequences for the amplification of *Dicrocoelium* spp. ITS-2 rDNA and mt-COX-1 mtDNA fragments.

rDNA nucleotide position	*D. dendriticum*	*D. chinensis*	COX-1 nucleotide position	*D. dendriticum*	*D. chinensis*
18	**T/A**	**T**	9	T	A
56	C	T	12	T	C
58	G	A	15	T	C
60	T	-	24	G	T
82	**T/C**	**T**	30	G	T
119	G	A	54	**T/C**	**T**
134	G	T	57	**T/A**	**T**
221	**C**	**C/A**	63	**T**	**T/C**
222	**T**	**T/A**	66	**G/A**	**G**
228	**A**	**A/G**	75	**T**	**A/T**
240	**A**	**A/G**	78	**C/T**	**T**
358	T	C	84	A	G
367	G	A	111	G	A
370	C	T	114	C	T
405	G	A	129	T	A
423	**C/T**	**T**	132	A	G
424	C	T	135	T	A
469	T	C	138	**T/C**	**T**
489	**T/C**	**T**	141	T	A
535	T	C	143	**T/G**	**T**
541	A	G	177	**C/T**	**T**
550	T	C	183	**T**	**C/T**
571	G	A			
630	T	A			
632	G	T			
654	T	A			
655	C	T			
671	**C**	**C/A**			
680	G	A			
681	T	G			
689	**G**	**A/G**			

Table 2. Sequence variation in a 698 bp fragment of rDNA and a 187 bp fragment of COX-1 mtDNA, differentiating between *D. dendriticum* and *D. chinensis*. The rDNA data are based on 12 sequences identified as *D. dendriticum* and 18 sequences identified as *D. chinensis* on NCBI GeneBank. The COX-1 data are based on 56 sequences identified as *D. dendriticum* and 11 sequences identified as *D. chinensis* on NCBI GeneBank. Informative sites to describe intraspecific variation are shown in bold.

rDNA haplotypes	Total number of sequences	Countries	mt-COX-1 haplotypes	Total number of sequences	Countries
*D. dendriticum*16	12	Pakistan	*D. dendriticum*1	4	Iran
*D. dendriticum*17	1	Pakistan	*D. dendriticum*2	2	Japan
*D. dendriticum*18	2	Pakistan, China	*D. dendriticum*3	2	Iran
*D. dendriticum*19	1	Pakistan	*D. dendriticum*4	33	Iran, China, Pakistan
*D. dendriticum*20	1	Pakistan	*D. dendriticum*5	2	Iran
*D. dendriticum*21	1	Pakistan	*D. dendriticum*6	19	Iran, China, Pakistan
*D. dendriticum*22	1	Pakistan	*D. dendriticum*7	7	Pakistan
*D. dendriticum*23	6	Pakistan, China	*D. dendriticum*8	1	Pakistan
*D. dendriticum*24	1	China			
*D. dendriticum*25	1	China			
*D. dendriticum*26	1	China			
*D. dendriticum*27	1	China			
*D. dendriticum*28	1	China			
*D. dendriticum*29	1	China			
*D. dendriticum*30	1	China			
*D. dendriticum*31	1	China			
*D. dendriticum*32	1	China			
*D. dendriticum*33	9	Pakistan			
*D. dendriticum*34	3	Pakistan			

Table 3. *D. dendriticum* rDNA and COX-1 haplotypes showing the number of sequences representing unique alleles and the country of origin. The accession numbers of all of the sequences are described in Supplementary Data S1 and S2 files.

The rDNA haplotype *D. dendriticum 18* represented two sequences from Pop-1 and Pop-7, which originated from the Booni and Mastuj regions and sequences reported from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 16* represented 12 sequences from Pop-2, Pop-3, Pop-4, Pop-6 and Pop-8, which originated from the Booni, Mastuj and Torkhow regions, while the closely related rDNA haplotypes *D. dendriticum 24, 25, 26, 27* and *28* represented sequences from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 23* represented 4 sequences from Pop-1 and Pop-5 which originated from the Booni and Laspoor regions, and sequences reported from the Shaanxi province of China. The rDNA haplotypes *D. dendriticum 19, 20* and *21* each represented single sequences from Pop-1 and Pop-2 which originated from the Booni region, while the closely related rDNA haplotypes *D. dendriticum 29, 31* and *32* represented sequences from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 33* represented 9 sequences from Pop-1, Pop-2, Pop-3 and Pop-5 which originated from the Booni and Laspoor regions. The rDNA haplotype *D. dendriticum 34* represented 3 sequences from Pop-1 and Pop-6 which originated from the Booni, and Mastuj regions. The rDNA haplotypes *D. dendriticum 17* and *22* represented single sequences from Pop-1 and Pop-6 which originated from the Booni and Mastuj regions, while the closely related rDNA haplotypes *D. dendriticum 25 and 30* represented sequences from the Shaanxi province of China.

The mtDNA haplotype *D. dendriticum 4* represented 32 sequences from Pop-2, Pop-3, Pop-5, Pop-6 and Pop-7, which originated from the Booni, Mastuj and Laspoor regions and sequences reported from the Shaanxi province of China and Shiraz province of Iran. This haplotype was closely related to *D. dendriticum 2* reported from Japan. The mtDNA haplotype *D. dendriticum 7* represented sequences from Pop-1, Pop-4, Pop-5, Pop-6 and Pop-8, which originated from the Booni, Mastuj, Laspoor and Torkhow regions. The mtDNA haplotype *D. dendriticum 8* represented one sequence from Pop-3, which originated from

the Booni regions. The mtDNA haplotype *D. dendriticum 6* represented 17 sequences from Pop-1, Pop-5 and Pop-6 which originated from the Booni, Mastuj and Laspoor regions and sequences reported from the Shaanxi province of China and Shiraz province of Iran.

Conflicts of interest

The authors declare no conflicts of interest related to this work.

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